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UNIVERSITÀ DEGLI STUDI DI TORINO

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Objectives

Post-operative scarring process on lumbar surgery is object of several studies mainly because of the epidural fibrosis formation. Hybrid chitosan have shown promising effect on fibrosis prevention. The aim of this study was to determine the influence of chitosan-silane membrane on the lumbar surgery scarring process. These membranes have improved mechanical strength which makes them suitable to maintain a predefined shape. Methods: A two level lumbar laminectomy was performed in 14 New Zealand male rabbits. Laminectomy sites were randomly selected for biomaterial or control. Chitosan membranes were prepared and care was taken in order to make it adapted to the bone defect dimensions covering the totality of the defect including the bone margins. Histological analysis was performed by haematoxylin/eosin and by Masson's trichrome staining four weeks after laminectomy. Results: Microscope observations revealed the presence of a well-organized regenerating tissue, integrated in the surrounding vertebral bone tissue with a regular and all-site interface on the chitosan sites, in clear contrast with the presence of a disorganized regenerating tissue with aspects consistent with the persistence of a chronic inflammatory condition, on control sites. Discussion: The results of this study clearly demonstrated that hybrid chitosan had an organizing effect on post-operative scarring process. The presence of the hybrid chitosan membrane resulted on a wellorganized tissue integrated in the surrounding vertebral bone tissue with signs of regenerative bone tissue in continuity with native bone. This can be a major feature on the dynamics of epidural fibrosis formation.

Keywords: Chitosan-silane, Epidural fibrosis, Hybrid chitosan, Laminectomy, Lumbar surgery

Introduction

Since the clarification of the sciatic pain pathophysiology, nearly a century ago, and its relationship to lumbar disk herniation, several surgical approaches were proposed and currently it is the most common indication for neurosurgical procedure in the US and there is evidence that the surgical treatment has advantage over conservative treatment.1–3 The surgical technique involves a posterior approach and an extradural disk removal.

Several authors have described different surgical techniques and a large majority describe gestures for protection against post-operative scarring.2,4–7 The prevention of post-operative perineural fibrosis has been object of increasing interest as this condition has been pointed out as one of the main causes of failed

back surgery syndrome.8–10 Ross et al.'s prospective, randomized, controlled, blind and multicentric trial, revealed a significant association between post-operative epidural fibrosis and recurrent radicular pain.11 Chitin is a co-polymer of N-acetyl-glucosamine and of N-glucosamine. When the number of Nacetyl-glucosamine units is superior to 50% it is called chitin and when the number of N-glucosamine units is superior that biopolymer is then called chitosan. Of these two biopolymers, chitosan has been more used on research due to its chemical properties. Chitin and chitosan are obtained from shellfish such as crabs and shrimps. It could be possible, on a near future, chitin or chitosan production through biotechnology techniques, especially if it is for medical applications.12 Advantageous physicochemical properties can be achieved by the cross-linking between chitosan, gamaglycidoxypropyltrimethoxysilane (GPTMS) and a siloxane network forming hybrid chitosan membranes.13–17

Materials and Methods

Animals For this in vivo study it was used 14 New Zealand male rabbits (Charles River, Barcelona, Spain). The animals' average weight was 3 kg. Each animal was submitted to a two level lumbar laminectomy (L1 and L3). For each animal, one laminectomy site was randomly selected for biomaterial application and the other laminectomy site acted as control. The animals were kept in the Veterinary Medical Teaching Hospital of the University of Tra´s-os-Montes e Alto Douro, and were fed with a standard rabbit regimen. All rabbits underwent a complete pre-operative neurological evaluation by a veterinary clinician to ensure complete neurological integrity. After surgery, all animals were kept under close surveillance for infection or neurological deterioration screening. All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). Hybrid chitosan membranes preparation Hybrid chitosan membranes were prepared by a previous described method.14 Chitosan (high molecular weight, Sigma, St. Louis, MO, USA) was dissolved in a 0.25M acetic acid solution to attain a concentration of 2% (w/v). The precursor sols were obtained by adding GPTMS (Sigma) to the chitosan solution. After stirring at room temperature for 1 hour, the resultant chitosan–siloxane solutions were poured into polystyrene containers and frozen at 220uC. The frozen sols were subsequently transferred to a freezedryer and then, they were lypophilized for 12 hours to complete dryness. The obtained porous hybrid xerogels were soaked in a 0.25M sodium hydroxide solution to neutralize the remaining acetic acid, and then washed with distilled water, and lypophilized again in the freeze-dryer. Before the implantation procedure the membranes were sterilized with ethylene oxide gas and kept at room temperature for 1 week. Surgical procedure Animals were anesthetized intravenously with ketamine (30 mg/kg) and medetomidine (0.1 mg/kg).

Then, lateral spine radiographs were performed in order to identify the number of lumbar vertebrae and, thus, localize the segments to operate. A midline incision exposed the spinal column at the L1–L3 level, and the paravertebral muscles were dissected bilaterally to visualize the transverse apophyses. The segments to surgically approach were identified intraoperatively by direct palpation. On each segment, the dorso-lumbar fascia was divided and a bilateral paravertebral muscles sub-periosteal dissection was performed. On this stage, the paravertebral muscles were retracted and it was then performed the bilateral laminectomy at L1 and L3 with a drill exposing the dura-mater. The laminectomy defects were measured to be approximately 1065 mm. In order to diminish the influence of any levelspecific variations the biomaterial implantation was placed on L1 laminectomy site (seven animals) or on L3 laminectomy site (seven animals) and the defect was left empty in the remaining site. Care was taken in order to produce a chitosan membrane adapted to the bone defect dimensions in a way that it covered the totality of the defect and also the bone of the margins (Fig. 1). A thorough haemostasis was performed in order to reduce the potential postoperative haematoma. Dorso-lumbar fascia was closed with simple stitches of resorbable

suture and the cutaneous layer was closed with intra-dermic continuous resorbable suture. The operative wound was cleaned with an iodopovidone solution. During the surgical procedure the animal's temperature, blood pressure and electrocardiogram were continuing monitored. An ophthalmic gel (Lacryvisc, Alcon, Lisbon, Portugal) was applied to prevent drying of the eyes. Postoperatively the animals were housed in individual cages and allowed normal activity. They were weighed daily for the first 7 days post-surgery and then weighed weekly. Post-operative care included injections of sulfadiazine and trimethoprim twice a day for up to 1 week. The animals were euthanized 4 weeks after laminectomy and the specimens were prepared for histological analysis.

Histological analysis

The vertebral L1–S1 segment was removed en bloc during the animals necropsy and was fixed on 10% formaldehyde. Decalcification of the entire vertebral segment was achieved using the Morse solution. In brief, decalcification was obtained using sodium citrate/formic acid until chemical testing for the presence of calcium runs negative; the samples were placed in 10 times their volume of the decalcifying solution and the solution was replaced daily. Chemical testing for the presence of calcium was carried out by adding 1 cc of ammonium hydroxide to 5 cc of the decalcifying solution plus 0.1 cc of a saturated ammonium oxalate solution. When calcium is still present in the solution, it precipitates, otherwise decalcification is complete (usually it takes around three weeks). The columns were then cut in 1 cm-long segments transversely to their axis. Each column was then rehydrated with phosphate buffer solution and cryoprotected with three passages in increasing solutions of sucrose (7.5% for 1 hour, 15% for 1 hour, 30% over-night) in 0.1M phosphate buffer solution. Thereafter, specimens are maintained in a 1:1 solution of sucrose 30% and optimal cutting temperature medium for 30 min and then embedded in 100% optimal cutting temperature medium. Specimens must then be stored at –80uC. Sections were then cut by means of a Leica CM1850 Cryostat in a thickness range of 20–30 mm, placed on silane-coated microscope slides to improve slice adhesion, and stored at –20uC. Before staining, sections were taken out of freezer to room temperature and as soon as they were acclimatized, they were further processed either by haematoxylin and eosin (the most commonly used stain for light microscopy observation in histology and histopathology) or by Masson's trichrome staining (that, in comparison to haematoxylin and eosin staining, it highlights also connective tissue). For haematoxylin and eosin staining, the slides were immersed in 0.1% haematoxylin (we use the product from Ciba, Basle, Switzerland) for 10 minutes, washed in tap water for 15 minutes, then immersed in 0.1% eosin (we use the product from Ciba, Basle, Switzerland) for 5 minutes and washed in distilled water. The sections were finally dehydrated in ethanol and mounted in DPX (Fluka, Buchs, Switzerland). For Masson's trichrome staining, we used a Masson trichrome with aniline blue kit (Bio-Optica, Milano, Italy): six drops of Weigert's iron haematoxylin

(solution A) and six drops of Weigert's iron haematoxylin (solution B) were combined together and used to stain slides for 10 minutes. Without washing, the slides were then drained and incubated with 10 drops of alcoholic picric acid solution for 4 minutes. After washing in distilled water, sections were stained with 10 drops of Ponceau acid fuchsin for 4 minutes and washed again in distilled water. Further on, 10 drops of phosphomolybdic acid solution were added to the section for 10 minutes. Without washing, the slides were drained and 10 drops of aniline blue are added to the section for 5 minutes. Finally, after washing in distilled water, dehydrating rapidly in ethanol and clearing in xylol/Bioclear (Bio-Optica, Milano, Italy), the slides were mounted in DPX (Fluka, Buchs, Switzerland). Sections were then analysed and photographed using a DM4000B microscope equipped with a DFC320 digital camera and an IM50 image manager system (Leica Microsystems, Wetzlar, Germany).

Results Results of the histological analysis of rabbit columns at the site of laminectomy are illustrated in Figs. 2–5. Extensive microscope observations, at different magnifications, showed, in the animals that did not receive chitosan application, the presence of a disorganized regenerating tissue (Fig. 2A, asterisk) mingled with islets of bone tissue (Fig. 2A, arrows). At higher magnification (Fig. 2B), the regenerating tissue appeared to be rich in various-sized and roundshaped cells (including many lymphocytes) that indicate the persistence of a chronic inflammatory condition. With respect to the bone tissue organization at the border of the lesion site, lamellae did not show osteonic organization and were oriented without clear orientation (Fig. 3A). Higher magnification observation (Fig. 3B) showed that the interface between the regenerating tissue and the bone tissue (Fig. 3B, arrow) has a limited extension without a clear orientation. Often, bone and regenerating tissue were completely detached and separated by large empty lacunae (Fig. 3A, asterisk). Histological analysis of rabbit columns at the site of laminectomy in the animals that received chitosan application are illustrated in Figs. 4 and 5. At lower magnification (Fig. 4), the presence of a well-organized regenerating tissue, integrated in the surrounding vertebral bone tissue though an interface (Fig. 4, arrows) that, contrary to what was observed in the control group, was regular and extended from the side to the other side of the lamina. The location of islets of bone tissue inside regenerating tissue (Fig. 5, arrows) and the small extension of regenerative tissue in comparison to controls suggest that bone regeneration has progressed significantly filling the lamina defect. Yet the presence of signs of osteonic reorganization (Fig. 4, asterisk) can be detected inside the regenerating tissue. However, since regenerated bone tissue can hardly be distinguished from native bone tissue, the amount of regenerated bone cannot be quantified.

Discussion

The epidural fibrosis formation is the result of postoperative haematoma invasion by thick fibrotic tissue, starting on the periosteum fibrous layer and fibroblast migration from the deep layer of the paravertebral muscles. This process can progress to the vertebral canal and create adherences to the duramater and nerve roots and inflammation is considered to play an important and early role in this process.18–20 Fibroblasts have multiple functions important to wound repair, such as collagen synthesis, extracellular matrix reorganization, and wound contraction resulting in mature scar formation. It has been shown that chitosan can inhibit fibroblast growth.21,22 Chang et al. in a study for reduction of peritoneal adhesions concluded that a chitosan barrier can inhibit tissue adhesion by inhibiting proliferation and triggering apoptosis.22 For this study it was used an animal laminectomy model using New Zealand rabbits. The bone anatomy of these animals is favourable for a lumbar laminectomy model as the dorsal laminae are long which makes the surgical procedure brief and straightforward, therefore reducing post-operative complications. One of the major complications of this procedure in rabbits is post-operative neurological deterioration which is more common with laminectomies on lower levels. This fact determined the choice of the laminectomy level for this work. Thus, in this study two different levels of lumbar laminectomy were performed, on L1 and L3. At these levels, on the vertebral canal, there is the lower portion of the spinal cord. Therefore, on this particular aspect, this model does not mimic the human lumbar laminectomy conditions. For these concerns, some authors have suggested lumbar laminectomy models in the region of the cauda equina (suggesting different animals, such as the ovine model) which would allow biomechanical testing on nerve roots.23,24 However, on the present work, the focus of the study was the histological characterization of the differences of post-operative fibrosis on a manipulated site (in the presence of the biomaterial) and on a control site. Therefore, the results are still valid and applicable to the biomaterial effects on post-operative epidural fibrosis. Another pitfall of the present study is that a simple laminectomy was performed but not a discectomy. Therefore, the effect that the experimental material might have on scar formation at discectomy site and on annular ligament healing could not be assessed.

The main reason for not performing the discectomy was the extreme difficulty to expose the nerve root and annulus fibrous without neurologic injury in a rabbit model. Chitosan matrices have been shown to have low mechanical strength under physiological conditions and to be unable to maintain a predefined shape for transplantation. The improvement of their mechanical properties can be achieved by modifying chitosan with a silane agent. The GPTMS is one of the silanecoupling agents, which has epoxy and methoxysilane groups. The epoxy group reacts with the amino groups of chitosan molecules while the methoxysilane groups are hydrolyzed and form silanol groups. The silanol groups are subjected to the construction of a siloxane network due to the condensation. Thus, the mechanical strength of chitosan can be improved by the cross-linking between chitosan, GPTMS and siloxane network.14 This hybrid chitosan membrane was used on the present work and was found to be very convenient to surgically manipulate in order to cover the entire bone defect. A synergistic effect of a more favourable porous microstructure and physicochemical properties (more wettable and higher water uptake level) of chitosan hybrids, and the presence of silica ions may be responsible for the good results in promoting posttraumatic nerve regeneration. In fact, chitosan hybrids were successful in improving sciatic nerve regeneration after axonotmesis.15,16 Other published studies revealed chitosan ability to promote cell membranes fusion after damage as chitosan is able to form large phospholipid aggregates and preferentially targets damaged tissues.25,26 On the other hand, in biocompatibility studies, hybrid chitosan membranes elicited a mild inflammatory response that decreased gradually, in clear contrast with the exuberant pyogranulomatous inflammatory reaction developed by non-hybrid chitosan membranes.16 One important aspect of the present study results was the finding that the hybrid chitosan membrane was consistently and totally degraded by the fourth week after the surgical procedure. This is an important feature as the barrier-effect for this biomaterial is important only in the early stages of the scarring process. Zhou et al. stressed this aspect on their work on reduction of post-surgical adhesion formation after cardiac surgery by application of N,O-carboxymethyl chitosan. Those authors described that the chitosan film used was effective on reducing post-surgical adhesion formation maintaining its structural integrity for five days and degrading by the seventh postoperative day.27 The results of this study clearly demonstrated that chitosan had an organizing effect on post-operative scarring process. The presence of the chitosan membrane resulted on a well-organized tissue integrated in the surrounding vertebral bone tissue. It was also observed signs of regenerative bone tissue in continuity with native bone which, by the present technique cannot be distinguished from each other. In fact, the four-week duration of this study allows only the important conclusion of improved vertebral regeneration on a post-laminectomy model. This can be a major feature on the dynamics of epidural fibrosis formation. Sandoval-Sa´nchez et al.28 achieved similar results using bilayer chitosan scaffolding as a dural substitute on experimental models. Chuang et al.,29 using a combination of poly-lactic acid gel and autologous micromorselized bone observed significant lamina bone regeneration.However,it is still unknown if the regenerated vertebral lamina can be expected to confer stability to the spinal column and prevent peridural adhesion as a long-term outcome. Further research on ligament structure will confirm whether a newly generated vertebral lamina can provide an

attached bed for soft tissue growth and enable ligament regeneration. Nonetheless, these studies are aiming tissue regeneration after surgical aggression which is significantly different from other current studies that purely aim inhibition of fibrosis such as the use of Mitomicin C.20,30 Those studies, chasing the same goal, use an entirely different approach and their results cannot be compared with results from studies using regenerative approaches. Whereas a quantitative analysis of the type or tissue repair was not possible due to its polymorph complexity, extensive microscopy observation carried out in the present study allowed to demonstrate that the morphology of tissue repair was rather consistent, in each of the two experimental groups, and rather differentiated between them. It can thus be concluded that hybrid

chitosan application after laminectomy improves vertebral regeneration by accelerating formation of a new and well organized cover tissue at the site of bone removal and by accelerating its quick differentiation into a newly formed and well organized bone tissue that integrates rapidly with the native bone filling the defect and recreating a vertebral structure which is close to the normal pre-lesion one. Unlike previous studies focused on inhibition of the scarring process in order to prevent epidural adhesions, the present work using this chitosan membrane, has showed that the scarring process can be directed to improving tissue regeneration suggesting a reduction on epidural fibrosis.

Disclaimer Statements

Contributors All authors contributed extensively to theworkpresentedinthis paper anddiscussedtheresults and implications and commented on the manuscript at all stages. Miguel Carvalho and Artur S. Vareja˜o: designed the experiment. Miguel Carvalho, Luı´s M. Costa and Jose´ E. Pereira: performed the neurosurgeries. Yuki Shirosaki, Satoshi Hayakawa, Jose´ D. Santos and Ana C. Maurı´cio: developed the Hybrid chitosan membranes. Stefano Geuna, Federica Fregnan and Anto´nio M. Cabrita: carried out the histological analysis. Artur S. Vareja˜o: supervised the project.

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Conflicts of interest None.

Ethics approval All procedures were performed with the approval of the Veterinary Authorities of Portugal in accordance with the European Communities Council Directive of 24 November 1986 (86/609/ EEC).

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